

Epithelial to Mesenchymal Transition Contributes to Drug Resistance in Pancreatic Cancer

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Abstract

A better understanding of drug resistance mechanisms is required to improve outcomes in patients with pancreatic cancer. Here, we characterized patterns of sensitivity and resistance to three conventional chemotherapeutic agents with divergent mechanisms of action [gemcitabine, 5-fluorouracil (5-FU), and cisplatin] in pancreatic cancer cells. Four (L3.6pl, BxPC-3, CFPAC-1, and SU86.86) were sensitive and five (PANC-1, Hs766T, AsPC-1, MIAPaCa-2, and MPanc96) were resistant to all three agents based on GI₅₀ (50% growth inhibition). Gene expression profiling and unsupervised hierarchical clustering revealed that the sensitive and resistant cells formed two distinct groups and differed in expression of specific genes, including several features of “epithelial to mesenchymal transition” (EMT). Interestingly, an inverse correlation between E-cadherin and its transcriptional suppressor, Zeb-1, was observed in the gene expression data and was confirmed by real-time PCR. Independent validation experiment using five new pancreatic cancer cell lines confirmed that an inverse correlation between E-cadherin and Zeb-1 correlated closely with resistance to gemcitabine, 5-FU, and cisplatin. Silencing Zeb-1 in the mesenchymal lines not only increased the expression of E-cadherin but also other epithelial markers, such as EVA1 and MAL2, and restored drug sensitivity. Importantly, immunohistochemical analysis of E-cadherin and Zeb-1 in primary tumors confirmed that expression of the two proteins was mutually exclusive ($P = 0.012$). Therefore, our results suggest that Zeb-1 and other regulators of EMT may maintain drug resistance in human pancreatic cancer cells, and therapeutic strategies to inhibit Zeb-1 and reverse EMT should be evaluated. [Cancer Res 2009;69(14):5820–8]

Introduction

Pancreatic cancer is the fourth leading cause of cancer death in the United States (American Cancer Society, 2007). Due to a lack of early detection methods and an absence of effective biomarkers, patients are usually diagnosed at a late stage with a five-year survival rate of <5%. Unfortunately, there remains no effective therapy available to treat this aggressive tumor. Although most chemotherapy regimens

use gemcitabine as the clinical standard of care for pancreatic cancer, patients generally have limited response to this therapy. Combination therapy and targeted therapies have also been overall quite disappointing. Thus, a better global understanding of the molecular mechanisms underlying drug resistance in pancreatic cancer may lead to the development of more effective therapeutic interventions.

Gene expression profiling has been used to identify biomarkers and therapeutic targets in pancreatic cancer (1, 2). In addition, identification of genes related to chemosensitivity in pancreatic cancer has been performed in an attempt to improve the efficacy of pancreatic cancer therapy, and several different biomarkers, including S100A4, S100P, BNIP3, Cox-2, and periostin, have been advanced as therapeutic targets (3–7). However, although there is an impression that pancreatic cancers tend to be cross-resistant to a large variety of cancer therapies, a mechanistic understanding of drug resistance has not been obtained.

Epithelial to mesenchymal transition (EMT) is a developmental process that seems to play an important role in tumor progression and metastasis in diverse solid tumors, including pancreatic cancer (8, 9). The EMT phenotype is characterized by the loss of cell-to-cell adhesion with the disintegration of tight, adherens, and gap junctions and a phenotypic change from an “epithelial” morphology to a motile, fibroblast-like morphology (10, 11). The hallmark of EMT is loss of the epithelial homotypic adhesion molecule E-cadherin and gain of mesenchymal markers such as vimentin and/or fibronectin. Suppression of E-cadherin expression in normal cells and cancers is mediated by several genetic factors, including mutation or promoter methylation of *CDH1*, or direct promoter repression mediated by Snail, Slug, Twist, Zeb-1, and Sip1 (10–13), all of which interact with E-box elements located within the proximal region of the E-cadherin promoter (10). Several clinical studies have shown that increased expression of E-cadherin is associated with improved survival in several tumor types (14, 15), and there is some evidence that small interfering RNA (siRNA)-mediated silencing of these transcriptional suppressors can increase cellular sensitivity to genotoxic stress (16).

Here, we characterized the effects of three conventional cancer chemotherapeutic agents on cell death in a panel of human pancreatic cancer cell lines to determine whether cells that were resistant to gemcitabine were also resistant to agents that act via independent mechanisms. We then used global gene expression profiling and unsupervised hierarchical clustering to show that the drug-resistant cells contained several features consistent with EMT. Reversal of EMT via silencing of Zeb-1 not only restored the expression of typical epithelial marker genes but also increased cellular sensitivity to therapeutic reagents. Our data suggest that Zeb-1 and other regulators of EMT maintain drug resistance in human pancreatic cancer cells, and therapeutic strategies to inhibit Zeb-1 and reverse EMT should be evaluated.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Materials and Methods

Cell lines and reagents. Ten pancreatic cancer cell lines were used for the generation of transcriptome data. Seven pancreatic cancer cell lines were obtained from the American Type Tissue Collection, including AsPC-1, MIAPaCa-2, PANC-1, BxPC-3, CFPAC-1, Hs766T, and SU86.86. MPanc96 and human pancreatic ductal epithelial (HPDE) cells were obtained from Dr. Timothy J. Eberlein (Washington University, St. Louis, MO) and Dr. M. Tsao (Ontario Cancer Institute, Toronto, Ontario, Canada), respectively. L3.6pl cells were derived from COLO357 that had undergone metastasis from the pancreas to the liver of nude mice (17). For the validation experiments, five cell lines (Suit2, SW1990, Capan-1, T3M4, and COLO357), which were generously provided by Drs. Eric Collisson, Joe Gray, and Martin McMahon (University of California and Lawrence Livermore Laboratory, San Francisco, CA), were used. All of the cell lines were genotyped by DNA fingerprinting (PowerPlex 16, Promega, Inc.). Gemcitabine was purchased from Eli Lilly and Co. 5-Fluorouracil (5-FU) and cisplatin were purchased from Sigma.

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay. Cells (3,000 per well) were plated in 96-well plate. After 24 h, cells were treated with increasing concentrations of the chemotherapy agents. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) solution (20 μ L; Promega) was added to each well and the cells were incubated at 37°C with 5% CO₂ for 1 h. Absorbance at 490 nm was then measured with a microplate reader (MRX, Danatech Laboratory).

Propidium iodide-fluorescence-activated cell sorting analysis. Standard propidium iodide (PI) staining by the hypotonic lysis method was used for apoptosis studies. Apoptosis was induced in 10⁶ cells by gemcitabine treatment. After 48/72 h, the cells were trypsinized, washed once with cold PBS, then incubated for 30 min in 500 μ L of hypotonic solution (0.1% sodium citrate, 0.1% Triton X-100, 100 μ g/mL RNase, and 50 μ g/mL PI), and analyzed by flow cytometry (Beckman Coulter, Inc.).

RNA isolation, microarray platform, and microarray experiments. All transcriptome data were generated from duplicates of the cell lines. Cells were plated and total RNA was isolated independently using Trizol reagent (Molecular Lab) followed by cleanup with RNeasy Mini kit (Qiagen). RNA was used for the synthesis of biotin-labeled cRNA, which was prepared using the Illumina RNA amplification kit (Ambion, Inc.), and then cRNA can be hybridized to Illumina Human-6v2 (Illumina, Inc.) chip. After being washed, the slides were scanned with BeadStation 500 \times (Illumina) and the signal intensities were quantified with BeadStudio (Illumina). Quantile normalization was used to normalize the data. Microarray data are available on Gene Expression Omnibus with accession number GSE15550.

Data processing. BRB ArrayTools version 3.6 developed by the National Cancer Institute (18) was used to analyze the data. To select genes that are differentially expressed between the two different subgroups (sensitive and resistant), a class comparison tool within BRB ArrayTools was used. The values were averaged over replicates of samples. This software uses a two-sample *t* test to calculate the significance of the observations (i.e., $P < 0.001$). To see global gene expression patterns, each gene values, adjusted to be a mean of zero, were used for unsupervised hierarchical clustering with Cluster and TreeView (19). The Pearson correlation coefficient was used to analyze the correlation between gene expression and chemosensitivity. Growth-inhibitory IC₅₀ (GI₅₀) values were calculated using GraphPad software.

Pathway analysis. Functional and pathway analysis was performed using the Ingenuity Pathway Analysis software. This software contains a database for identifying networks and pathways of interest in genomic data.

Real-time PCR analysis. Real-time PCR technology (StepOne, Applied Biosystems) was used in conjunction with Assays-on-Demand (Applied Biosystems). The comparative C_T method was used to determine relative gene expression levels for each target gene and cyclophilin A served as an internal control to normalize for the amount of amplifiable RNA in each reaction.

Silencing of Zeb-1 expression by siRNA. Dharmacon SMARTpool control and Zeb-1 siRNA were used with Oligofectamine according to the manufacturer's protocol (Invitrogen Corp.). Cells were incubated with the

siRNA complex for 48 h, then treated with chemotherapeutic reagents, and harvested for mRNA and protein expression changes [assayed via real-time reverse transcription-PCR (RT-PCR) and Western blot] at 48 h. Cell death was measured by PI-fluorescence-activated cell sorting (FACS) analysis.

Western blotting. Total cellular protein extract was isolated from harvested cells, protein concentration was determined, and Western blotting was carried out as described previously (20). The antibodies used were anti-Zeb-1 (Santa Cruz Biotechnology, Inc.), anti-E-cadherin (Zymed Laboratories, Inc.), and anti-actin (Sigma Chemical Co.). Same antibodies were used for immunohistochemistry.

Immunohistochemistry. Cells grown in chambered slides were washed with cold PBS and fixed with iced acetone for 5 min at room temperature. Cells were washed with PBS and blocked with blocking solution (horse serum/goat serum, 1:4) for 30 min. After cells were incubated with anti-E-cadherin/Zeb-1/vimentin (Santa Cruz Biotechnology) overnight at 4°C, cells were washed with PBS and incubated with FITC-conjugated secondary antibody (1:200; Santa Cruz Biotechnology) for 1 h. Cells were washed with PBS and mounted with Prolong Gold antifade reagent with 4',6-diamidino-2-phenylindole (Invitrogen).

For the human samples, unstained pancreatic tissue microarray (TMA) slides were deparaffinized with xylene and rehydrated with ethanol. Antigen retrieval was carried out by repetitive boiling and cooling cycles for a total of 15 min in antigen unmasking solution (Vector Laboratories). Endogenous peroxidase activity was blocked with 6% hydrogen peroxide in methanol, and nonspecific binding sites were blocked with normal donkey serum. Primary antibody diluted (1:50) in 2% bovine serum albumin/0.2% Triton in PBS was added, and samples were incubated overnight at 4°C, after which biotinylated secondary antibody was added and incubated for 30 min followed by Vectastain Elite avidin-biotin complex method reagent (Vector Laboratories) and incubation for an additional 30 min. Finally, slides were developed with 3,3'-diaminobenzidine, counterstained with hematoxylin, dehydrated with ethanol, and fixed with xylene and mounted. The staining results were evaluated by a pathologist to determine the intensity and the percentage of positive tumor cells. The staining for Zeb-1 was categorized as positive (nuclear staining in $\geq 10\%$ of tumor cells) or negative (no nuclear staining or nuclear staining in $< 10\%$ of tumor cells). The staining for E-cadherin was categorized as 0 (negative or less than 10% moderate to strong membranous staining in tumor cells), 1 ($\geq 10\%$, but less than 50% moderate to strong membranous staining), or 2 ($\geq 50\%$ moderate to strong membranous staining). The correlation between Zeb-1 and E-cadherin was analyzed by Fisher's exact tests using Statistical Package for the Social Sciences software for Windows (version 12.0; SPSS, Inc.). We used a two-sided significance level of 0.05.

Results

Characterization of pancreatic cancer cells based on multidrug sensitivity. We examined the native sensitivity of one immortalized "normal" human pancreatic epithelial line (HPDE) and nine commonly available pancreatic cell lines to gemcitabine *in vitro* by PI-FACS analysis. All of the lines were genotyped by DNA fingerprinting using a commercial kit. Five of the lines (HPDE, L3.6pl, BxPC-3, CFPAC, and SU86.86) were sensitive and five (PANC-1, Hs766T, AsPC-1, MIAPaCa-2, and MPanc96) were resistant based on the level of apoptosis (sub-G₀-G₁ population) stimulated with 10 μ mol/L gemcitabine (Fig. 1A). To test the multidrug sensitivity of those cell lines, we performed cell viability assays using two additional chemotherapeutic agents: cisplatin and 5-FU. Interestingly, all of the gemcitabine-sensitive cancer cell lines were substantially more sensitive to 5-FU and cisplatin compared with the gemcitabine-resistant lines (Fig. 1B and C). Log₁₀(GI₅₀) values of a panel of cell lines and cluster analysis using log₁₀(GI₅₀) values were plotted (Fig. 1D). In the sensitive cells, log₁₀(GI₅₀) values were below the mean log₁₀(GI₅₀) across the panel, whereas resistant cell lines had log₁₀(GI₅₀) values above the mean.

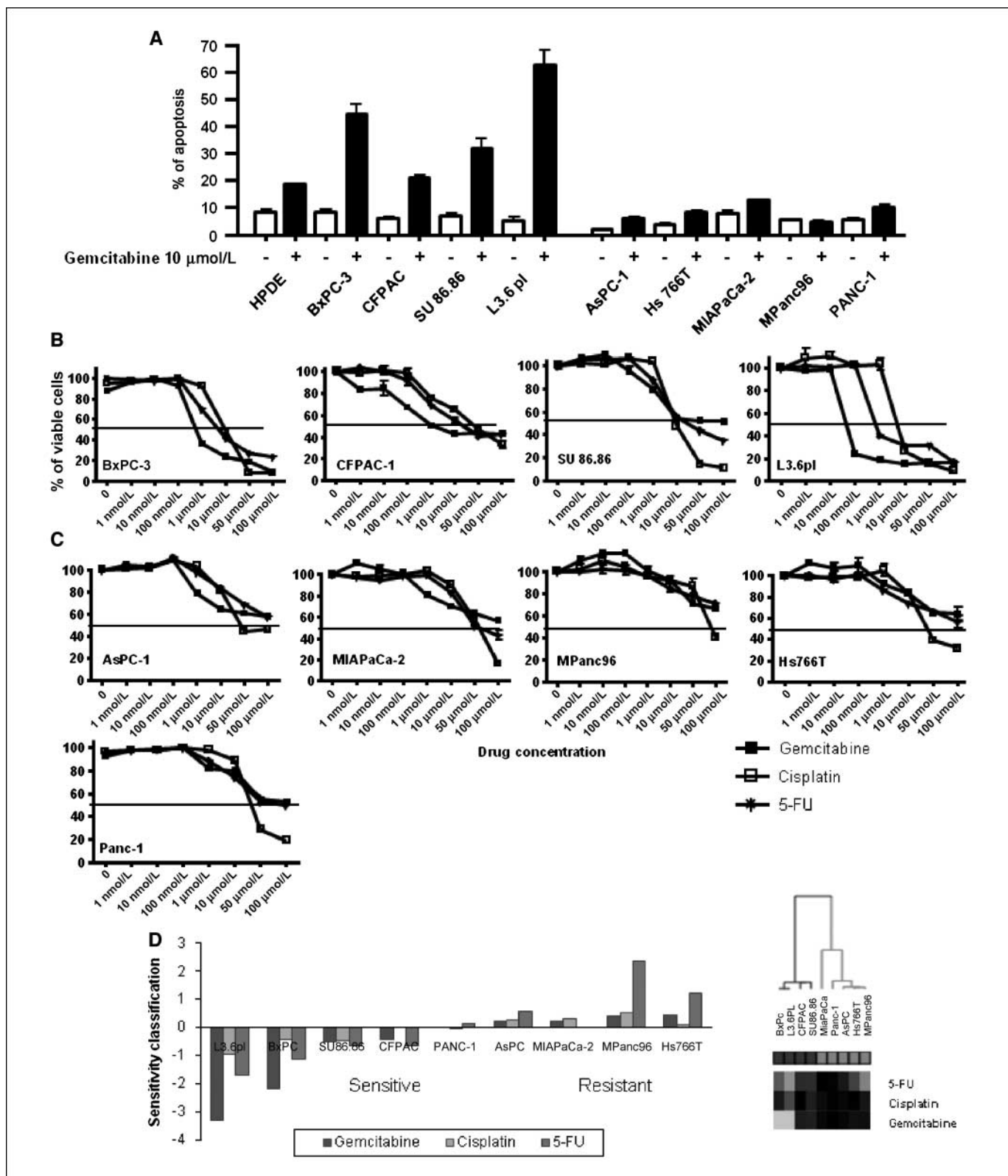


Figure 1. Effect of chemotherapeutic drugs on pancreatic cancer cells. *A*, proapoptotic effects of gemcitabine on pancreatic cancer cells. Pancreatic cancer cells (1.0×10^6) were seeded in culture plates and incubated with and without 10 $\mu\text{mol/L}$ gemcitabine. After 72 h, apoptotic cells were measured by FACS analysis. Columns, mean of three experiments; bars, SE. *B* and *C*, effects of various chemotherapeutic drugs on pancreatic cancer cell viability. Cells were treated with increasing concentrations of gemcitabine, 5-FU, and cisplatin (1–100,000 nmol/L). After 72 h, viable cells were quantified using the MTS reagent. Points, mean of three experiments; bars, SE. *D*, $\log_{10}(\text{GI}_{50})$ values and cluster analysis of using $\log_{10}(\text{GI}_{50})$ values were plotted. If $\log_{10}(\text{GI}_{50})$ of the cell lines were below the mean of $\log_{10}(\text{GI}_{50})$ across the panel of cells, the cell lines were defined as sensitive, whereas resistant cell lines had $\log_{10}(\text{GI}_{50})$ values above the mean.

Identification of global gene expression patterns of pancreatic cancer cells. Our results suggested that drug sensitivity and resistance might be controlled by broad-based mechanism(s). In an attempt to elucidate these mechanisms, we performed global gene

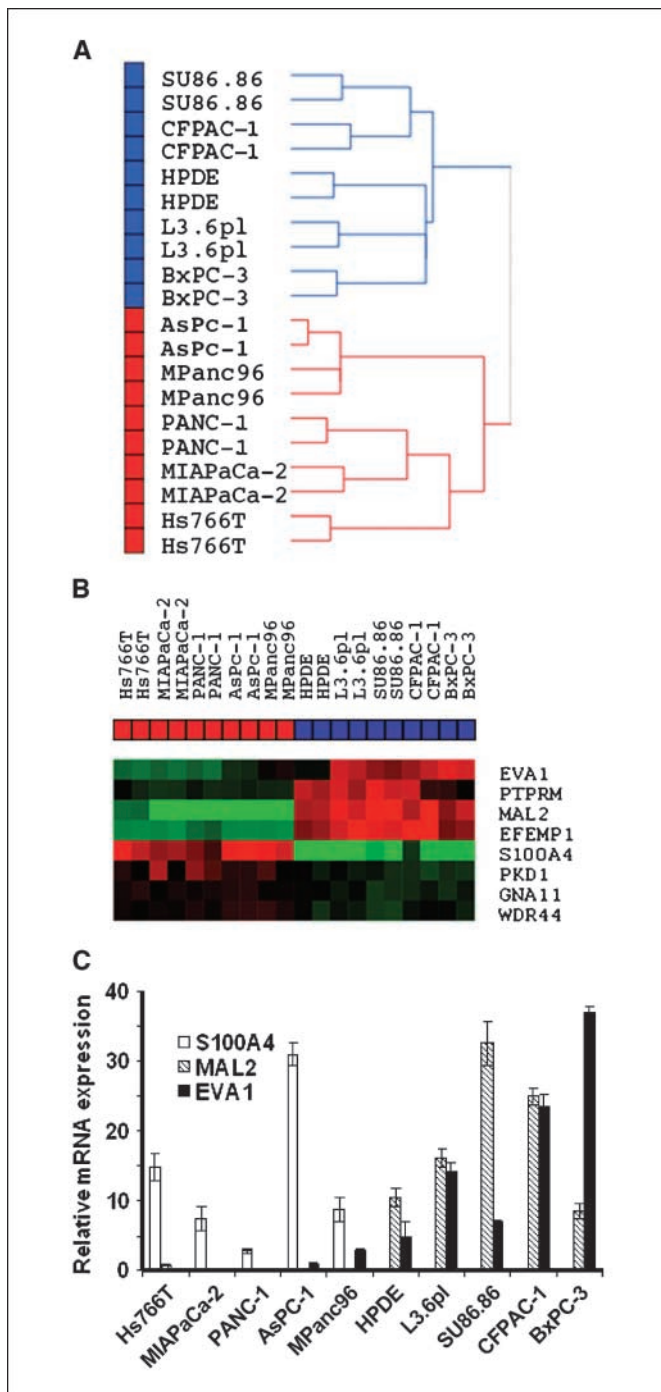


Figure 2. Differentially expressed genes between sensitive and resistant cells. **A**, unsupervised hierarchical clustering using whole-genome expression patterns in the array (average linkage clustering). Cell lines are separated as two distinct subgroups that correlated with drug sensitivity. *Blue box*, sensitive cells; *red box*, resistant cells. **B**, EMT-related genes with significant differences in expression between sensitive and resistant cells ($P < 0.001$). *Red*, highly expressed genes; *green*, lower expressed genes. **C**, the differential expression of MAL2, S100A4, and EVA1 was confirmed by real-time PCR. *Columns*, mean of triplicate samples; *bars*, SE.

expression profiling on the nine pancreatic cancer cell lines and the normal HPDE cells using duplicate mRNA isolates obtained at different times. Unsupervised hierarchical clustering using whole-genome expression patterns in the array showed two distinct subgroups that clearly separated the drug-sensitive from the drug-resistant cell lines (Fig. 2A). Clustering of the 6,127 genes that were differentially expressed in at least two arrays with at least a 2-fold change showed the same expression pattern as the whole genome (Supplementary Fig. S1). This unsupervised clustering analysis revealed two subgroups that correlated with the multidrug sensitivity data identified in the proliferation and apoptosis assays in Fig. 1 (Fig. 2A).

Differential expression of cellular adhesion and motility molecules. To identify genes that may play a role in drug sensitivity/resistance, the differentially expressed genes between sensitive and resistant cell lines were extracted using the class comparison tool in BRB ArrayTools ($P < 0.001$). We identified several interesting networks/pathways that were statistically significantly enriched, including those involved in cellular movement, cellular development, molecular transport, and cancer using Ingenuity Pathway Analysis (Supplementary Table S1). The 115 genes that were used for the pathway analyses are listed in Supplementary Table S2 ($P < 0.001$). However, one of the most obvious patterns that emerged from these analyses involved genes that had been previously implicated in EMT, including genes required for cell polarity (*MAL2* and *RABGEF1*), adherens junction formation (*EVA1* and *PTPRM*), and cell motility (*S100A4*, *GNA11*, *EFEMP1*, *WDR44*, and *PKD1*; Fig. 2B). All genes in Fig. 2B had statistical significance at the cutoff of $P < 0.001$, except *EVA1* ($P = 0.0025$). The differential expression of MAL2, S100A4, and EVA1 was confirmed by real-time PCR (Fig. 2C), and P values and fold changes of the genes are summarized in Table 1. In addition to t test, to determine the relationship between drug sensitivity and gene expression, Pearson correlation analysis was performed using GI_{50} values (in Fig. 1D) and expression of genes in Fig. 2B (Supplementary Table S3). Not all the expression of the genes was directly correlated with GI_{50} , although the significant patterns were observed in class comparison analysis (t test). We speculate that the EMT phenotype is roughly bimodal in the cells, which may explain why gene expression and GI_{50} are not linearly correlated.

Cellular morphology and the expression of EMT markers. We subsequently performed experiments to assess the role of EMT in drug resistance in more detail. Simple light microscopic analysis of the cell lines confirmed that the drug-sensitive cells were uniform in shape and grew in tightly adherent “sheets” of cells in monolayer culture, whereas the drug-resistant cells were more irregular in shape and did not form close attachments in culture (data not shown), indicating that the drug-resistant cells displayed a more “mesenchymal” phenotype. To further confirm our observation, we assessed the levels of E-cadherin (epithelial marker; $P = 0.0084$), Zeb-1 (a transcriptional suppressor of E-cadherin; $P = 0.0085$), and vimentin (mesenchymal markers; $P = 0.12$) in the gene expression data. We observed a statistically significant inverse correlation ($r = -0.797$; $P = 0.006$) between E-cadherin and Zeb-1 (Fig. 3A) that was associated with the EMT phenotype. We confirmed these expression data by real-time RT-PCR using the same RNA from the array experiment (Fig. 3A). We then analyzed the protein levels of epithelial marker E-cadherin and its transcriptional suppressor Zeb-1 and also the mesenchymal marker vimentin in three sensitive and three resistant cells by immunofluorescence staining (Fig. 3B–D). The staining patterns

Table 1. Adhesion and motility molecules with significant difference in expression between sensitive and resistant cells

	PTPRM	WDR44	GNA11	EVA1	EFEMP1	MAL2	S100A4	PKD1
<i>P</i>	0.00035	0.00066	0.00076	0.00256	2.1e-06	7.9e-06	4.75e-05	0.00067
Fold changes (S/R)	3.98	-1.64	1.55	4.95	13.33	40.21	-35.16	-2.12

Abbreviation: S/R, sensitive cells/resistant cells.

clearly confirmed the gene expression and real-time RT-PCR data in that all of the epithelial cell lines expressed E-cadherin with no expression of Zeb-1 and vimentin, whereas all the resistant cell lines highly expressed Zeb-1 with no expression of E-cadherin and vimentin, with the exception of Hs766T, which does not express vimentin. To test the hypothesis that mesenchymal cells would show more migratory properties, we performed migration assays. As we expected, in general, the epithelial cells were less migratory than the mesenchymal cells (Supplementary Fig. S2).

Effects of silencing Zeb-1 on EMT-related gene expression and drug sensitivity. The gene expression and real-time PCR data suggested to us that Zeb-1 might negatively regulate E-cadherin expression and possibly drug sensitivity. To test this hypothesis, we knocked down Zeb-1 using siRNA in PANC-1 and assessed the effects on EMT-related gene expression. Zeb-1-specific siRNA significantly reduced expression of Zeb-1 and that this was associated with a significant induction of E-cadherin mRNA and protein expression (Fig. 4A and B). Furthermore, Zeb-1 silencing up-regulated expression of EVA1 and MAL2, two other epithelial markers that were expressed at low levels in the mesenchymal cells, showing that all three epithelial genes are directly or indirectly regulated by this transcriptional repressor (Fig. 4B). Then, we measured the effects of Zeb-1 silencing on drug-induced cell death in several of the drug-resistant cell lines (PANC-1, MIAPaCa-2, and Hs766T). Significant increases of apoptotic cell death were measured in Zeb-1 silencing cells after gemcitabine, 5-FU, and cisplatin treatment in PANC-1 and MIAPaCa-2, whereas moderate effects were observed in Hs766T, showing that Zeb-1 does play a direct role in drug resistance in pancreatic cancer cells (Fig. 4C).

Validation of the inverse correlation of E-cadherin and Zeb-1 expression in primary patient tissues and five independent pancreatic cancer cell lines. Qualitative analyses of E-cadherin and Zeb-1 expression in a pancreatic patient TMA revealed an inverse correlation between Zeb-1 and E-cadherin expression ($P = 0.012$). E-cadherin was identified primarily in the cell junctions as an adherent molecule and Zeb-1 was found primarily in the nucleus as a transcriptional factor (Fig. 5A).

To further evaluate the relationship between the inverse correlation between E-cadherin and Zeb-1 and drug sensitivity, we performed an independent validation experiment using five new pancreatic cancer cell lines. Two cell lines (Suit2 and SW1990) expressed high-level expression of Zeb-1 and low-level expression of E-cadherin, whereas the other three cell lines (Capan-1, T3M4, and COLO357) showed high expression of E-cadherin with low expression of Zeb-1 (Fig. 5B). As anticipated, the epithelial cells (Capan-1, T3M4, and COLO357) were relatively sensitive to gemcitabine, cisplatin, and 5-FU, and opposite trend was observed in mesenchymal cells (Suit2 and SW1990; Fig. 5C). Expression profiling of four of five cell lines (T3M4, COLO357, Capan-1, and

Suit2) further confirmed our hypothesis, showing that both groups clustered along with sensitive or resistant subtypes in Fig. 2A (Supplementary Fig. S3). COLO357, the parental cell line for L3.6pl, expressed high levels of E-cadherin and low levels of Zeb-1 and was highly sensitive to drug, showing that the phenotype is stable even after multiple *in vitro* and *in vivo* passaging of the cells.

Discussion

In this study, we identified a molecular mechanism of multidrug resistance in pancreatic cancer cells. We evaluated the sensitivity of a panel of pancreatic cancer cells to three chemotherapeutic agents and identified a correlation between the patterns of drug sensitivity/resistance and global gene expression. Importantly, Zeb-1-mediated EMT seems to be a major mediator of this drug resistance in pancreatic cancer cell lines.

Our global gene expression patterns show that there are shared drug sensitivity and resistance mechanisms in pancreatic cancer (Fig. 2A). Morphologic changes identified by light microscopy led us to examine the expressional correlation of E-cadherin and its transcriptional regulators in our transcriptome analysis. The loss of E-cadherin expression mediated by transcriptional suppression has been associated with a poor clinical outcome in several types of cancers (14, 21). The importance of particular transcriptional repressors in maintaining the EMT phenotype may vary depending on the tissue type (14, 15, 21–24). However, our pharmacogenomics approach suggests that Zeb-1 plays a dominant role in the pancreatic cancer cell lines we studied here and that Snail, Slug, Twist, and Sip1 do not correlate significantly with drug resistance (Supplementary Fig. S4). We confirmed that the inverse correlation between E-cadherin and Zeb-1 was also present in primary patient tumor samples (Fig. 5A). Whether EMT correlates with gemcitabine resistance in patients will require further investigation.

A recent study using siRNA to silence Zeb-1 showed that Zeb-1 can suppress the transcription of multiple genes involved in determining epithelial polarity, including the cadherin families and components of tight and gap junctions, showing the ubiquitous role of the EMT phenotype genes (25). Similarly, we have shown that increased E-cadherin expression after silencing of Zeb-1 was accompanied by increased drug sensitivity (Fig. 4C). Importantly, EMT also correlates with resistance to the epidermal growth factor receptor (EGFR) inhibitor erlotinib (26), a biological agent that is currently being combined with gemcitabine in pancreatic cancer patients (27), and our data show that Zeb-1 silencing also enhances sensitivity to erlotinib in PANC-1 cells.⁶ Zeb-1 represses E-cadherin

⁶ A. Kwan, unpublished data.

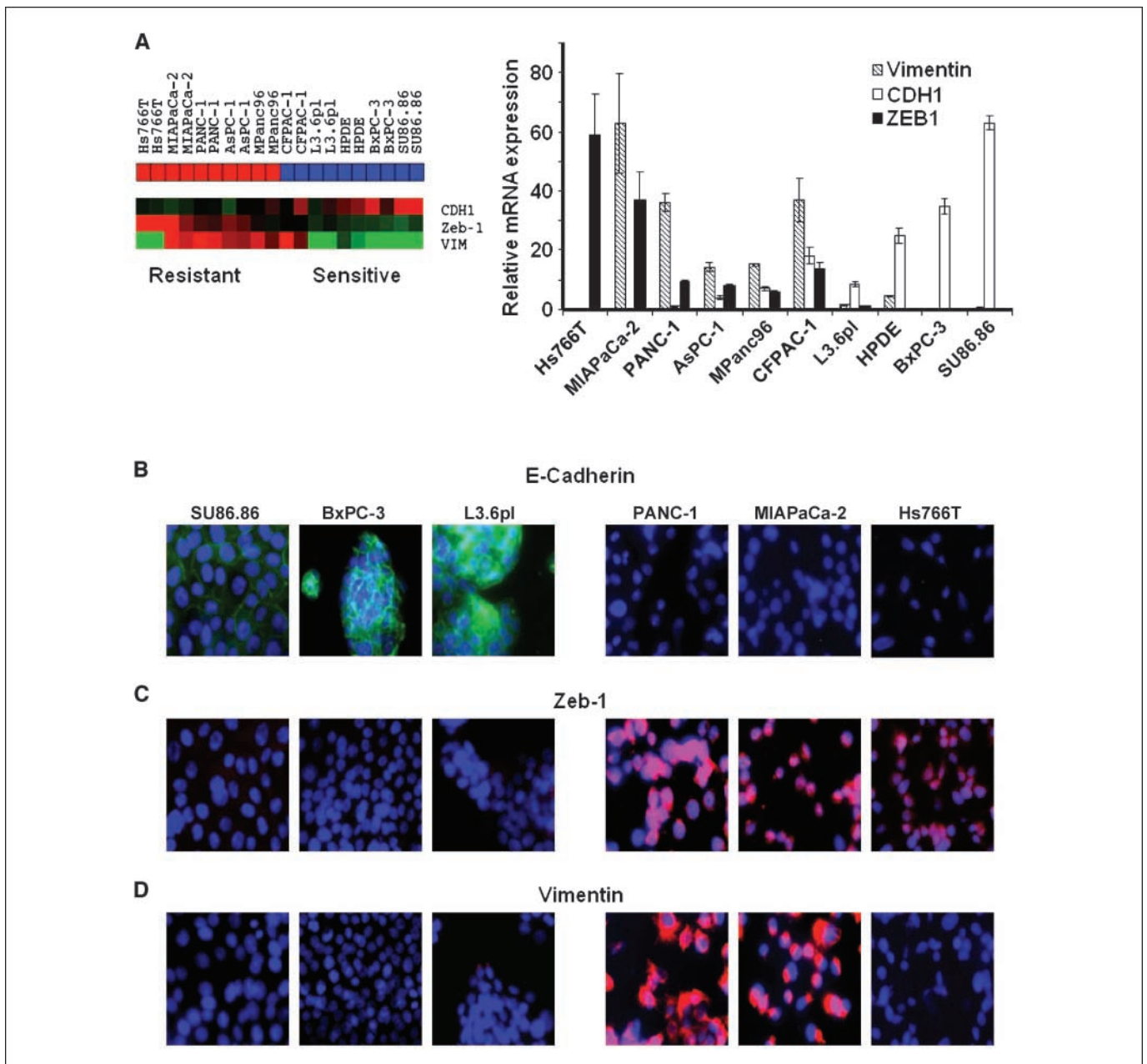


Figure 3. Expression of EMT markers in pancreatic cancer cells. *A*, expression patterns of E-cadherin, Zeb-1, and vimentin in the array data were generated via heat map and confirmed by quantitative real-time PCR. An inverse correlation between E-cadherin and Zeb-1 was observed across the cell lines. *Columns*, mean of triplicate samples; *bars*, SE. *B* to *D*, immunofluorescence localization of E-cadherin, Zeb-1, and vimentin confirms the association of epithelial and mesenchymal phenotype in drug sensitivity and resistance.

expression by recruiting histone deacetylases (HDAC) to E-box elements within the E-cadherin promoter. Thus, recent studies have shown that clinically relevant HDAC inhibitors, such as suberoylanilide hydroxamic acid (SAHA), can restore E-cadherin expression and sensitivity to gemcitabine and other agents (28, 29). We have also found that SAHA causes up-regulation of E-cadherin and down-regulation of Zeb-1 and restores gemcitabine and gefitinib sensitivity in several of the mesenchymal lines characterized in this study.⁷ Thus, it may be possible to use HDAC inhibitors

to reverse the EMT phenotype and restore drug sensitivity to pancreatic cancers and other solid tumors.

All of the drugs used in this study are DNA damaging agents. Among commonly used conventional cancer chemotherapeutic agents, taxanes are unique because they do not seem to act by inducing DNA damage but rather target microtubules leading to mitotic arrest and apoptosis. For reasons that are not entirely clear, taxanes have not been used for pancreatic cancer treatment. We tested the effects of paclitaxel in our panel of cell lines to determine whether EMT correlated with Taxol resistance. Interestingly, paclitaxel resistance did not correlate well with EMT (data not shown). For example, the GI₅₀s of paclitaxel in the Hs766T and

⁷ K.F. Fournier and A. Kwan, in preparation.

MIAPaCa-2 cells were lower than the mean GI_{50} across the panel (Supplementary Fig. S5). It has been reported that paclitaxel sensitivity does not correlate with sensitivity to cisplatin and oxaliplatin in primary human pancreatic cancer cells (30). In future studies, we plan to explore the molecular basis for the observed heterogeneity in taxane responsiveness in our cells. It is possible that taxanes might have activity in pancreatic cancers that are resistant to the agents that are currently being used in the treatment of the disease.

Gemcitabine efficacy is limited due to the rapid development of resistance in patients with pancreatic cancer. There are multiple known factors responsible for this resistance, including up-regulation of phosphatidylinositol 3-kinase/AKT, S100A4, HMGA, and the ATP-binding cassette (ABC) transporter genes (3, 31, 32). With respect to the latter, 48 distinct transporters within seven different subfamilies have been identified. They function as

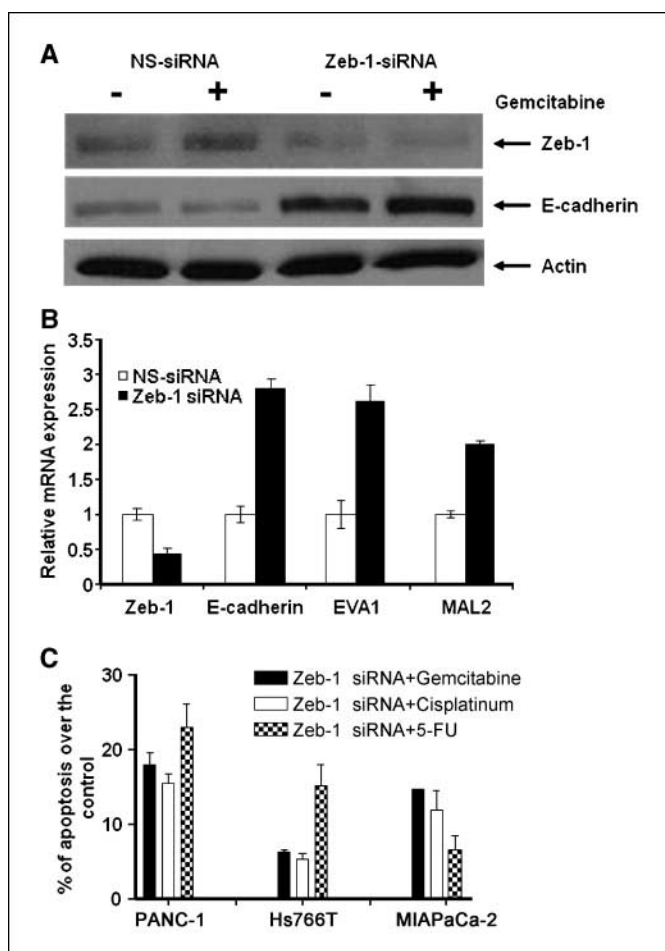


Figure 4. Effects of silencing Zeb-1 on EMT-related gene expression and drug sensitivity. *A*, cells were transiently transfected with Zeb-1-specific siRNA or nonspecific control for 48 h, and the expression of Zeb-1 and E-cadherin protein was measured in PANC-1 cells. *B*, the mRNA expression of Zeb-1, E-cadherin, EVA1, and MAL2 was measured after Zeb-1 silencing. Zeb-1-specific siRNA significantly reduced the expression of Zeb-1 and this was associated with a significant induction of E-cadherin, EVA1, and MAL2 mRNA levels. *Columns*, mean of triplicate samples; *bars*, SE. *C*, effects of Zeb-1 silencing on drug sensitivity in the resistant cells. Zeb-1-specific siRNA or nonspecific control-transfected PANC-1, MIAPaCa-2, and Hs766T cells were incubated with 10 μ mol/L gemcitabine, 5-FU, and cisplatin for up to 72 h, and then apoptotic cells were measured by PI-FACS analysis. Data converted into percentage of apoptosis over the control.

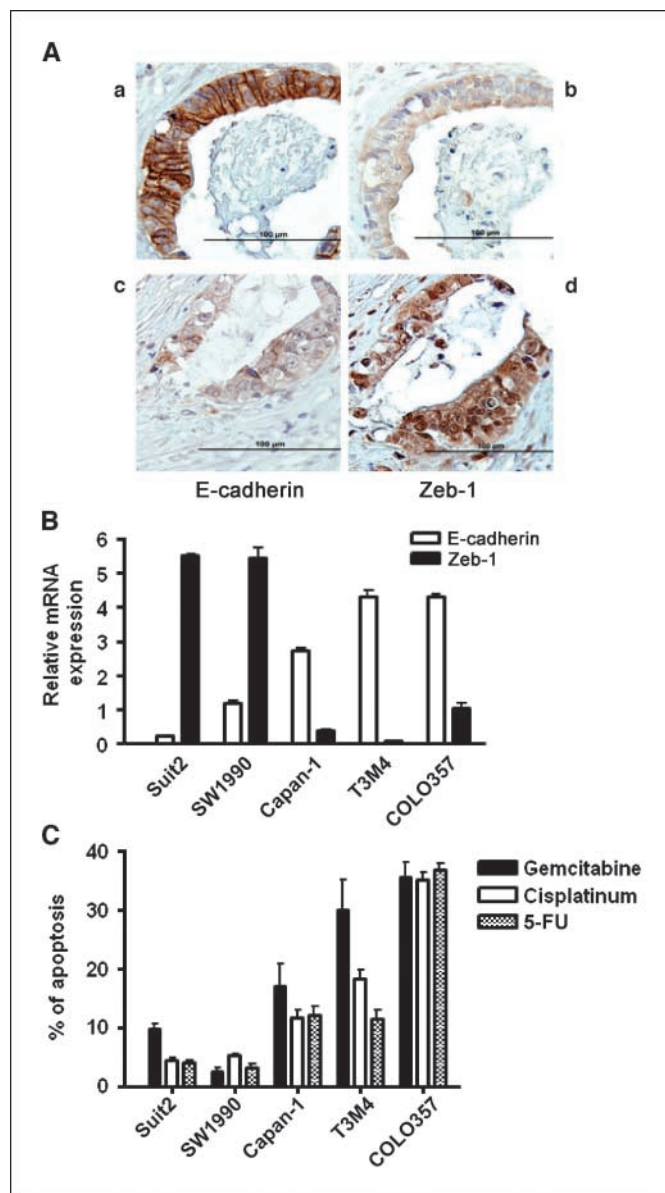


Figure 5. *A*, expression of E-cadherin and Zeb-1 in primary patient tumors. *a* and *b*, E-cadherin-positive cancer cells are negative for Zeb-1. *c* and *d*, Zeb-1-positive cells are negative for E-cadherin. *B*, the inverse correlation between E-cadherin and Zeb-1 expression in independent validation experiment. *C*, apoptotic effects of gemcitabine, cisplatin, and 5-FU in a validation set of five new cell lines measured by PI-FACS analysis.

transmembrane proteins that transport lipid, cholesterol, and drug metabolites out of the cell. It has been shown that several of these ABC transporters are associated with gemcitabine resistance (32, 33). Interestingly, we did not detect any relationship between the expression of ABC transporters and drug sensitivity within our panel of cells (Supplementary Fig. S6). S100A4, one of the calcium binding protein family of S100s, has been linked to migratory and invasive properties in several other solid tumors, including prostate cancer, breast cancer, and gastric cancer (34–36). We also confirmed that the resistant cells with high expression of S100A4 have greater migratory properties than the sensitive cells that have a low expression of S100A4 (Supplementary Fig. S2). Knockdown of S100A4 also has been reported to cause an increase in E-cadherin

expression and an increased latency of tumor formation (34). Conversely, increased expression of S100A4 and decreased expression of E-cadherin have been shown to correlate with a poor prognosis in pancreatic cancer patients (36). Evaluation of patients samples has shown hypomethylation of S100A4, causing down-regulation of E-cadherin in the majority of pancreatic cancers. This shows its role as a potential prognostic marker and possible therapeutic target (37, 38). Our study confirms the inverse relationship between S100A4 and E-cadherin as well. However, the mechanism of regulation of S100A4 and E-cadherin is not clear, and further investigation of this mechanism will be required.

The loss of cellular polarity and homotypic adhesion are major components of EMT. Our transcriptome analyses revealed various EMT-related genes, such as *PKD*, *MAL2*, and *EVA1*, which seem to be associated with drug sensitivity in pancreatic cancer cells. Transforming growth factor- β (TGF- β) is a known activator of the EMT program via the major signaling pathways Smad, raf, notch, and PKD (39–41). The blockage of PKD expression by siRNA or a specific inhibitor attenuated TGF- β -induced EMT (41). Likewise, *EVA1* and *MAL2* are expressed on many types of epithelial cells (42–44). The functions of *EVA1* and *MAL2* in human cancer are not well characterized, but their mRNA expression levels correlated with sensitivity to EGFR inhibitors and the epithelial phenotype (26, 45). Interestingly, knockdown of Zeb-1 expression restored the expression of *MAL2* (46). Our data also identify an inverse correlation between *EVA1/MAL2* and Zeb-1 expression in pancreatic cancer cells (Fig. 4B), again suggesting an important role of Zeb-1 in regulating the EMT program.

In this study, the measurement of basal gene expression levels enabled us to identify Zeb-1-mediated EMT as a multidrug resistance mechanism in pancreatic cancer. Zeb-1 silencing can partially restore the drug sensitivity in resistant cells, implying that other signaling pathways might be responsible for drug resistance mechanism. Because of the limited number of cell lines used in our experiments, there may be many other important biological factors that contribute to drug resistance in pancreatic cancer. Furthermore, pancreatic cancer is exemplified by the presence of an extensive tumor-associated stroma, and recent studies have confirmed that stromal cells also contribute to drug resistance (47). We are currently expanding our panel of cell lines and models to include conventional and primary xenografts that directly address these issues. Overall, we remain confident that by using gene expression profiling as a starting point to identify the biological properties associated with drug sensitivity or resistance, we will ultimately be able to better match tumors to effective therapies and improve disease control in patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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